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Note

Lack of relationship between genotype and virulence in *Candida* species

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ABSTRACT

Background: The virulence of isolates among different *Candida* species causing candidemia may play a role in the prognosis of the patients. Furthermore, the potential relationship between genotype and virulence is still unclear and need to be further studied.
Aims: We aim to assess the relationship between genotype and virulence in *Candida* species using a *Galleria mellonella* larvae infection model.
Methods: One hundred and ninety-four isolates from 68 clusters (*Candida albicans*, 114/41; *Candida parapsilosis*, 74/24; *Candida tropicalis*, 6/3) were compared against the same number of each species singleton genotypes in terms of survival of *G. mellonella* larvae.
Results: The median of survival and the IQR ranges of clusters and singleton were as follows: *C. albicans* (2 days, IQR 1.5–2 *vs.* 2 days, IQR 1–2.25), *C. parapsilosis* (2 days, IQR 1.5–2.6 *vs.* 2 days, IQR 2–3.3), and *C. tropicalis* (1 day, IQR 1–3.5 *vs.* 2 days, IQR 2–3.5; $p < 0.05$). High intra-cluster variability in terms of median of survival was found regardless the species.
Conclusions: No relationship between genotype and virulence in *Candida* was observed with the *G. mellonella* model.

Ausencia de relación entre el genotipo y la virulencia en especies de *Candida*

Palabras clave:
Candida albicans
Candida parapsilosis

RESUMEN

Antecedentes: La virulencia de cepas de diferentes especies de *Candida* causantes de candidemia puede jugar un papel en el pronóstico de los pacientes, y su estudio en el modelo de infección en *Galleria mellonella* puede ser útil para entender su contribución general a la infección. Además, la potencial relación entre genotipo y virulencia requiere de más estudios.

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Candida tropicalis
Genotipado
Galleria mellonella
Virulencia

Objetivos: Se evaluó la relación entre genotipo y virulencia en especies de *Candida* mediante el modelo de infección de larvas de *G. mellonella*.
Métodos: Se estudió la supervivencia de las larvas infectadas con 194 aislados incluidos en 68 *clusters* (*Candida albicans*, 114/41; *Candida parapsilosis*, 74/24; *Candida tropicalis*, 6/3) y con el mismo número de aislados con genotipos únicos por especie.
Resultados: La mediana de supervivencia y los rangos intercuartílicos (IQR) de *clusters* y genotipos únicos se muestra a continuación: *C. albicans* (2 días, IQR: 1,5-2 *vs.* 2 días, IQR: 1-2,25), *C. parapsilosis* (2 días, IQR: 1,5-2,6 *vs.* 2 días, IQR: 2-3,3), y *C. tropicalis* (un día, IQR: 1-3,5 *vs.* 2 días, IQR: 2-3,5; $p < 0{,}05$). Encontramos una importante variabilidad en la mediana de supervivencia entre cepas del mismo *cluster*, independientemente de la especie analizada.
Conclusiones: No se encontró relación entre el genotipo y la virulencia entre los aislados de *Candida* evaluados mediante el modelo de infección de *G. mellonella*.

Candidemia is primarily caused by *Candida albicans*, *Candida parapsilosis*, *Candida glabrata*, and *Candida tropicalis*.[5] The virulence of *Candida* isolates, such as adherence and biofilm formation, the ability to evade the host's immune system, and the production of tissue-damaging hydrolytic enzymes (proteases, phospholipases, and haemolysins), may play a role in the prognosis of infected patients.[10,11] *Galleria mellonella* has become a useful model of infection for testing different pathogens as larvae are easy to handle, are cheap, and their innate immune system resembles that of mammals.[14] However, the relationship between genotype and virulence is mostly unknown.[1,8,9,12,13] We previously showed intra- and inter-species differences in terms of virulence among *Candida* strains using a *G. mellonella* infection model,[8] and that clusters present greater biofilm production than singleton isolates.[3] Here we evaluated the potential relationship between genotype and virulence in *Candida* by studying *Candida* cluster and singleton genotypes using a *G. mellonella* model.

We studied 68 clusters of *Candida* species causing candidemia in patients admitted to 16 tertiary hospitals in Spain, Denmark, Italy and Brazil during 2014–2015. Isolates had been genotyped previously using species-specific microsatellite markers.[3,4] A cluster was defined as an identical genotype infecting at least two patients. Comparisons of 194 isolates from clusters (*C. albicans*, $n = 114$ isolates from 41 clusters; *C. parapsilosis*, $n = 74$ isolates from 24 clusters; *C. tropicalis*, $n = 6$ isolates from 3 clusters) and other 194 isolates with singleton genotype (one isolate per genotype) from patients with candidemia admitted to Gregorio Marañón hospital, were carried out. Virulence was established by assessing the survival in the *G. mellonella* larvae infection model as previously reported.[8] Briefly, each *Candida* inoculum was adjusted to 5×10^7 cells/ml (*C. albicans* and *C. tropicalis*) or 2×10^8–5×10^8 cells/ml (*C. parapsilosis*) with a Neubauer hematocytometer. Each experimental group containing 10 larvae of *G. mellonella* (Bichosa, Salceda de Caselas, Spain) with similar size and weigh were infected with 10 μl of each cell suspension. Ten larvae PBS-inoculated and 10 non-injected larvae were used as controls. Larvae were incubated at 37 °C, and death was determined visually for seven days. Isolate survival curves were generated using Kaplan–Meier method. Median survival was calculated and log-rank (Mantel–Cox test) was applied to examine the differences between the survival curves (Graph Pad Prism statistical 5.02 software, GraphPad, La Jolla, USA). The interquartile range (IQR) of median survival was calculated per species, and for clusters and singletons, by pooling the isolates. Isolates were further classified as highly virulent (median survival < percentil$_{25}$), low virulent (median survival > percentile$_{75}$) and moderately virulent (in between) according to the pooled analysis of clusters and singleton isolates. This study was approved by the Ethics Committee of Hospital Gregorio Marañón (CEIC-A1; study no. 201/18).

PBS control larvae and those non-injected were alive the seven days post-infection. Overall, the pooled median survival times of larvae infected by *Candida* species were the following: *C. albicans* (2 days, IQR 1–2), *C. tropicalis* (2 days, IQR 1.13–3.13), and *C. parapsilosis* (2 days, IQR 2–3). Differences in median survival between larvae infected by cluster isolates and singletons did not reach statistical significance except for *C. tropicalis* (Fig. 1; $p < 0.05$). Mean of survival and IQR ranges of clusters and singleton were as follows: *C. albicans* (2 days, IQR 1.5–2 *vs.* 2 days, IQR 1–2.25; $p > 0.05$), *C. parapsilosis* (2 days, IQR 1.5–2.6 *vs.* 2 days, IQR 2–3.3; $p > 0.05$), and *C. tropicalis* (1 day, IQR 1–3.5 *vs.* 2 days, IQR 2–3.5; $p < 0.05$). High intra-cluster variability in terms of mean survival was found regardless the species. Based on median survival data, clusters *vs.* singleton isolates were classified as highly virulent [*C. albicans* (0% *vs.* 0%), *C. parapsilosis* (28.4% *vs*. 10.8%), and *C. tropicalis* (50% *vs.* 0%)], moderately virulent [*C. albicans* (76% *vs.* 75.4%), *C. parapsilosis* (48.6% *vs.* 64.9%), and *C. tropicalis* (16.7% *vs.* 86.3%)], and low virulent [*C. albicans* (3.7% *vs.* 24.6%), *C. parapsilosis* (23% *vs.* 24.3%), and *C. tropicalis* (33.3% *vs.* 16.7%).

This study supports previous observations of higher mortality associated to *C. albicans* and *C. tropicalis* in comparison to *C. parapsilosis* in both *G. mellonella* models and clinical observations.[5,8] Moreover, we aimed to decipher potential intra-species differences in terms of virulence between clusters and singletons. Differences reached statistical significance only for *C. tropicalis,* although the low number of isolates due to the lower number of cases of candidemia caused by this species ($n = 12$) did not allow us to reach solid conclusions.[4,5] We carried out a species-specific classification of isolates based on *G. mellonella* mortality. Subtle differences were observed, despite the fact that we found significantly higher percentages of highly virulent isolates of *C. parapsilosis* and *C. tropicalis* in clusters compared with singletons. These observations suggest that genotypes and mortality in *G. mellonella* are unrelated. In previous studies, contradictory conclusions were reported when the genotype was compared with virulence factors not related to *G. mellonella* mortality. Some authors found positive correlations,[6,9,12,15] and others did no find such relationship.[1,7,13] One of the most studied virulence factors is biofilm formation. *C. albicans* biofilm producing isolates are more aggressive than non-biofilm producer isolates in the *G. mellonella* larvae model,[2] although we were unable to initially support these observations in a previous study.[8] Subsequently, we included genotyping in the equation and observed a tendency of *C. albicans* clusters to form more biofilm than singletons.[3] However, the huge intra-cluster variability found in terms of biofilm formation,[3] along with the variability in terms of mortality in *G. mellonella* reported

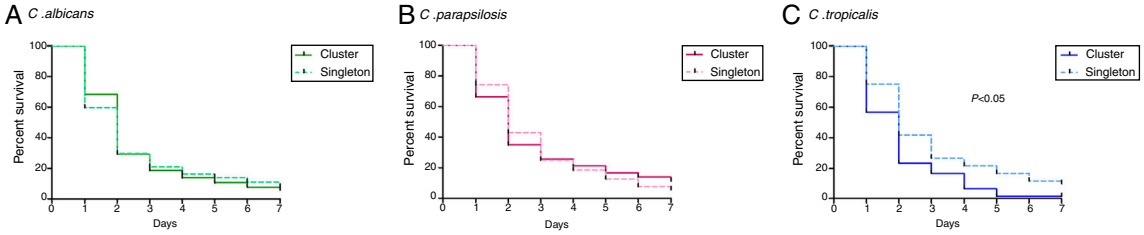

Fig. 1. Survival curves of *G. mellonella* larvae infected with *C. albicans* (a), *C. parapsilosis* (b), and *C. tropicalis* (c) isolates (clusters and singleton genotypes).

here, suggest notable isolate-to-isolate differences rather than a genotype–phenotype virulence relationship. Our study is subject to certain limitations. First, we have a heterogeneous number of isolates per species. Second, since whole genome sequencing of these isolates has not yet been performed, the variability among isolates in clusters can be a consequence of the lack of discriminatory potential for the microsatellites. Finally, we were unable to include any clinical data of patients and make a correlation between our *in vitro* findings and patient outcome.

In conclusion, no relationship between genotype and virulence in *G. mellonella* infection model was found for the three studied *Candida* species, suggesting that epigenetic factors are important determinants of virulence.

Author contributions

Judith Díaz-García: methodology; formal analysis; writing – original draft preparation. Maiken C. Arendrup and Rafael Cantón: data collection; resources (samples); writing –review & editing. Julio García-Rodríguez, Elia Gómez García de la Pedrosa, Gabriella Parisi, Javier Pemán, Brunella Posteraro, Maurizio Sanguinetti, Daniel Archimedes Da Matta, Arnaldo L. Colombo, Patricia Muñoz, Carlos Sánchez-Carrillo: data collection; resources (samples) and writing. Jesús Guinea and Pilar Escribano: conceptualization; project administration; data collection; supervision; validation; visualization; writing – original draft preparation and review & editing.

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Conflict of interest

JG has received funds for participating at educational activities organized on behalf of Astellas, Gilead, Pfizer, MSD, Scynexis, and Biotoscana-United Medical; he has also received research funds from FIS, Gilead, Scynexis, Cidara, Basilea Ltd, and F2G outside the submitted work. ALC received educational grants from Biotoscana-United Medical, MSD, Pfizer and a research grant from Astellas and Pfizer. RC has received funds for participating at educational activities organized by MSD and Pfizer. MCA reports personal fees from Astellas, Basilea, Gilead, MSD, Pfizer, T2Biosystems, and Novartis, and others from Amplyx, Astellas, Basilea, Gilead, T2Biosystems, F2G, Novabiotics and Scynexis outside the submitted work. The rest of the authors have no conflicts of interests.

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